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Abstract: Normal physiologic functions of the cellular prion protein (PrPc) are still elusive. This GPI-anchored protein exerts many functions, including roles in neuron proliferation, neuroprotection or redox homeostasis. There are, however, conflicting data concerning its role in synaptic transmission. Although several studies report that PrPc participates in NMDA-mediated neurotransmission, parallel studies describe normal behavior of PrPc-mutant mice. Abnormal axon connections have been described in the dentate gyrus of the hippocampi of PrPc-deficient mice similar to those observed in epilepsy. A study indicates increased susceptibility to kainate (KA) in these mutant mice. We extend the observation of these studies by means of several histologic and biochemical analyses of KA-treated mice. PrPc-deficient mice showed increased sensitivity to KA-induced seizures in vivo and in vitro in organotypic slices. In addition, we show that this sensitivity is cell-specific because interference experiments to abolish PrPc expression increased susceptibility to KA in PrPc-expressing cells. We indicate a correlation of susceptibility to KA in cells lacking PrPc with the differential expression of GluR6 and GluR7 KA receptor subunits using real-time RT-PCR methods. These results indicate that PrPc exerts a neuroprotective role against KA-induced neurotoxicity, probably by regulating the expression of KA receptor subunits.

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Enhanced Susceptibility of *Prnp*-Deficient Mice to Kainate-Induced Seizures, Neuronal Apoptosis, and Death: Role of AMPA/Kainate Receptors

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Normal physiologic functions of the cellular prion protein (PrPc) are still elusive. This GPI-anchored protein exerts many functions, including roles in neuron proliferation, neuroprotection or redox homeostasis. There are, however, conflicting data concerning its role in synaptic transmission. Although several studies report that PrPc participates in NMDA-mediated neurotransmission, parallel studies describe normal behavior of PrPc-mutant mice. Abnormal axon connections have been described in the dentate gyrus of the hippocampi of PrPc-deficient mice similar to those observed in epilepsy. A study indicates increased susceptibility to kainate (KA) in these mutant mice. We extend the observation of these studies by means of several histologic and biochemical analyses of KA-treated mice. PrPc-deficient mice showed increased sensitivity to KA-induced seizures in vivo and in vitro in organotypic slices. In addition, we show that this sensitivity is cell-specific because interference experiments to abolish PrPc expression increased susceptibility to KA in PrPc-expressing cells. We indicate a correlation of susceptibility to KA in cells lacking PrPc with the differential expression of GluR6 and GluR7 KA receptor subunits using real-time RT-PCR methods. These results indicate that PrPc exerts a neuroprotective role against KA-induced neurotoxicity, probably by regulating the expression of KA receptor subunits.

Key words: neuronal excitotoxicity; cellular prion protein; kainate binding receptors; epileptic seizure

The cellular prion protein (PrPc) is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein present in mammals, birds, and several invertebrates. PrPc, encoded by the *Prnp* gene in mammals, is highly expressed by neurons and glial cells in the adult central nervous system (CNS) (Kretzschmar et al., 1986; Moser et al., 1995; Brown et al., 1997a; Fournier, 2001; Ford et al., 2002; Moleres and Velayos, 2005). The abnormal processing of PrPc gives rise to a proteinase-k resistant isoform termed PrPsc, which is the etiologic agent of several transmissible spongiform encephalopathies (Prusiner, 1982). These encephalopathies are characterized by profound histologic changes, including extensive neuronal death, reactive gliosis and neuroinflammation, and extracellular accumulation of aggregated PrPsc in infected brains (Prusiner, 1998a,b; Prusiner et al., 1998; Aguzzi and Polymenidou, 2004).

Although many studies have examined the function of PrPc, its physiologic role in healthy neurons remains to be elucidated. PrPc was implicated initially in copper homeostasis in healthy brain (Brown et al., 1997a; Vassallo and Herms, 2003). Other studies have reported PrPc to be involved in additional neural functions such as neuronal plasticity (Maglio et al., 2004) and proliferation (Steele et al., 2006), or neurite outgrowth (Chen et al., 2003; Santuccione et al., 2005). Interestingly, recent studies point to a neuroprotective role of PrPc (Chiarini et al., 2002; Chen et al., 2003). Indeed, PrPc is overexpressed in ischemic brain and correlates with neuroprotective effects (McLennan et al., 2004; Weise et al., 2004). Moreover, *Prnp* −/− hippocampal cultures

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are more susceptible to cell death than *Prnp* +/+ neurons after serum removal (Kuwahara et al., 1999). Finally, PrPc protects human primary neurons in culture against bax-mediated apoptosis (Bounhar et al., 2001), thus corroborating the role of this protein in neuroprotection and cell survival.

The first descriptions of mice lacking the *Prnp* gene reported intrinsic resistance to prion infection (Bueler et al., 1993) but also normal development and performance in most behavioral tests (Bueler et al., 1992). However, further detailed studies proposed that the phenotype of *Prnp* −/− neurons is particularly sensitive to stress (Brown et al., 1997b, 2002), which correlates with alterations in sleep and circadian impairment in PrPc-deficient mice (Tobler et al., 1997, 1996).

Prnp −/− mice display mossy fiber collateral and terminal sprouting in the hippocampus, similar to observations in epileptic patients (Colling et al., 1997). In addition, PrPc-mutant mice show increased susceptibility to and death after kainate (KA) injections compared to wild-type animals (Walz et al., 1999). It has been reported that glutamate treatment is less toxic than KA in *Prnp* −/− neurons (Brown et al., 2002). These differences may be associated with differential expression of glutamate/KA receptors or with affinity variables. Little data is available about these issues and a correlation has been shown only recently between the increased long-term potentiation (LTP) observed in the hippocampus of *Prnp* −/− mice with the overexpression of mRNAs for *N*-methyl-D-aspartate (NMDA) receptor subunits NR2A and NR2B in *Prnp* −/− neurons (Maglio et al., 2004). No detailed biochemical study of AMPA/KA receptor expression or histologic description of susceptibility of *Prnp* −/− mice to KA have been carried out. We show, using several methods, the cell-specific susceptibility of hippocampal *Prnp*-deficient neurons to KA in vivo as well as in vitro in organotypic cultures. Indeed, loss-of-function experiments in neuroblastoma cells using siRNA against PrPc increased susceptibility to KA in transfected cultures. Finally, we show that this susceptibility correlates with the differential expression GluR6 and GluR7 KA receptors in *Prnp*-deficient neurons.

MATERIALS AND METHODS

Animals

PrPc-deficient mice (*Prnp* −/−) were purchased from EMMA (Monterotondo, Italy). Forty-four *Prnp* −/− adult mice were used. In addition, 21 C57BL6J mice (Iffa Credo, France) were also used. To avoid putative background-specific differences between *Prnp* and wild-type mice, *Prnp* −/− mice were crossed with C57BL6J mice from several generations and the experiments were conducted using littermates derived from heterozygous (*Prnp* +/−) parents (50 littermates, 34 adult mice and 16 newborn mice). Genotyping of *Prnp* −/− mice was done using two PCR reactions, as described by Bueler et al. (1993) (see also Gavin et al., 2005 for details). All experimental procedures were carried out in accordance with the guidelines of the Spanish Ministry of Science and Technology following European standards.

KA Injections and Scoring of Seizure Severity

The C57BL6J strain is seizure-resistant in comparison to other genetic backgrounds of mice (McKhann et al., 2003). The *Prnp* −/− strain used in the present study was generated on a C57BL6J background (Bueler et al., 1992). Thus, to induce convulsive non-lethal seizures in C57BL6J mice, we developed a progressive KA treatment by administering several consecutive intraperitoneal (i.p.) injections of the glutamate agonist KA (Sigma, Poole Dorset, UK) dissolved in 0.1 M phosphate buffered saline (PBS) pH 7.4. A total of 10 *Prnp* +/+, 13 *Prnp* −/− mice, and 5 *Prnp* +/− were subjected to KA experiments. *Prnp* +/+, *Prnp* +/−, and wild-type animals were processed in parallel. Animals were weighed and i.p. injected with several pulses (four injections) of KA (8 mg/kg b.w.) every 30–60 min for up to 4 hr. Injected animals displayed forelimb clonus, rearing, and falling, or continuous tonic clonic seizures and were analyzed during 7 hr after the first injection. Seizure intensity after KA injections was evaluated as described previously (Peng et al., 1997; Lee et al., 2000). After the first KA injections, the animals developed profound hypoactivity and immobility (Grade I–II). After successive injections, hyperactivity (Grade III) and scratching (Grade IV) were often observed. Some animals (especially *Prnp* −/− mice) progressed to a loss of balance control (Grade V) and further chronic whole body convulsions (Grade VI). The so-called "popcorn" bouncing activity was included in Grade VI of the scale used in our study. After the behavioral study, mice were kept in separate boxes until histologic or biochemical study 48 hr after the treatment. Twelve hours after KA-treatment, wild-type and *Prnp* +/− mice showed normal behavior compared to *Prnp* −/−. KA-treated *Prnp* −/− mice displayed hypoactivity and immobility. This hypoactivity was paralleled by relevant sensitivity of mice to external stimuli (e.g., box handling). These low activity symptoms were observed in *Prnp* −/− mice until processing.

Organotypic Slice Cultures of Hippocampus

Hippocampal slice cultures ($n = 50$) were prepared from P0 (day of birth), P1 *Prnp* +/+ ($n = 10$), or mutant mouse pups ($n = 10$), as described previously (Del Rio et al., 1997). Animals were anaesthetized, brains were removed aseptically from the skull, and hippocampi were dissected. Horizontal slices (325–350 μm thick) containing the hippocampus were then prepared using a McIlwain tissue chopper (Mickle Laboratory, Surrey, UK). Sections were maintained and selected in Minimum Essential Medium (MEM) supplemented with glutamine (2 mM) (MEM dissecting salt solution) for 45 min at 4°C. Selected slices were then cultured using the membrane interface method (Stoppini et al., 1991). Slices were placed on 30-mm diameter sterile membranes (Millicell-CM, Millipore, Billerica, MA) and transferred to six-well tissue culture plates (Nunc, Roskilde, Denmark). Cultures were incubated with 1.2 ml of culture medium (50% MEM; 25% horse serum; 25% HBSS) containing 2 mM glutamine and 0.04% $NaHCO_3$ adjusted to pH 7.3. The membrane cultures were maintained

in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The medium was changed every 2 days. All culture reagents were purchased from Invitrogen-Life Technologies (Merelbeke, Belgium).

Pharmacologic Treatments of Slice Cultures

Neuronal death after glutamate or KA treatment in organotypic slice cultures was monitored by propidium iodide (PI) uptake (Del Rio et al., 1997; Noraberg et al., 2005). Hippocampal slice cultures were deprived of serum for 3 hr and exposed to 50 μM of KA for 2 hr. After treatment, cultures were rinsed twice in KA-free culture media. The next day, slices were incubated for 2 hr with 1 μg/ml of PI dissolved in culture media. PI-treated slices were fixed with 2% paraformaldehyde dissolved in 0.1 M PBS (pH = 7.3) for 1 hr. After rinsing in 0.1 M PBS, cultures were mounted in Mowiol, examined, and photo documented in a confocal microscope. To determine the max/min PI fluorescence for quantification, parallel time-aged cultures were treated with 50 mM glutamate for 2 hr or alternatively with 0.1 M PBS, and processed as above. Total fluorescence in the pyramidal layer of the hippocampus was measured in a 500-μm segment of the layer in the CA1-3 regions using Quantity One Imaging and Analysis software (BioRad, Hertfordshire, UK). In parallel cultures, the non-competitive NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,b]-cyclohepten-5,10-imine (MK-801; Sigma, St. Louis, MO) (Wong et al., 1986) was added 30 min before KA-treatment at 10 and 30 μM to *Prnp* −/− cultures to address the participation of NMDA receptors in the KA-induced cell death.

Histologic Methods

Two days after KA-treatment, mice (control and KA-treated) were perfused with phosphate buffered 4% paraformaldehyde pH 7.3, postfixed overnight in the same fixative, and cryoprotected in 30% sucrose. Coronal sections (30-μm thick) were obtained in a freezing microtome. Free-floating sections from different mice were processed in parallel. Free-floating sections were rinsed in 0.1 M PBS and endogenous peroxidases were blocked by incubation in 3% H_2O_2 and 10% methanol dissolved in 0.1 M PBS. After extensive rinsing, sections were incubated in 0.1. M PBS containing 0.2% gelatin, 10% normal serum, 0.2% glycine, and 0.2% Triton-X 100 for 1 hr at room temperature. Sections were then incubated overnight at 4°C with the distinct primary antibodies, using the following dilutions: α-fos (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA); α-GluR1, (1:1,000, Chemicon, Temecula, CA); α-GluR2/3, (1:500, Chemicon); α-GluR4, (1:250, Chemicon); α-GluR6-7, (1:250, Chemicon); α-ERK1-2 pthreonine-202/ptyrosine-204, α-p38 pthreonine180/ptyrosine182 or JNK-pthreonine-183/ptyrosine-185 (all 1:200, Cell Signalling, Beverly, MA); and glial fibrillary acidic protein (α-GFAP, 1:200, Dakopatts, Denmark). Thereafter, sections were incubated with secondary biotinylated antibodies and streptavidin–horseradish peroxidase complex (Vector Labs, Burlingame, CA), following the manufacturer's instructions (Vector Labs) Sections were developed with 0.025% diaminobenzidine and 0.003% hydrogen peroxide, mounted onto slides, dehydrated, and coverslipped with Eukitt (Merck, Darmstadt, Germany). Selected sections were processed for double immunofluorescence (e.g., pERK1-2 + GFAP) using Alexa-Fluor 488- and Alexa-Fluor 568-tagged secondary antibodies (Molecular Probes, Eugene, OR).

Fluoro-Jade B Staining of Dying Neurons in Brain Sections

Coronal brain sections were obtained as above. Thereafter, sections were rinsed for 2 hr in Tris 0.1 M, pH 7.4, mounted, and air-dried at room temperature overnight. The next day, sections were pre-treated for 3 min in absolute alcohol, followed by 1 min in 70% ethanol and 1 min in distilled water. They were then oxidized in a solution of 0.06% $KMnO_4$ for 15 min. After three rinses of 1 min each with distilled water, the sections were incubated for 30 min in a solution of 0.001% Fluoro-Jade B (Chemicon) containing 0.01% of DAPI in 0.1% acetic acid. The slides were rinsed in deionized water for 3 min each, dried overnight, cleared in xylene, and coverslipped with Eukitt. Sections were examined using an epifluorescent microscope with blue-violet excitation light set at 450 nm and 350 nm, respectively. Fluoro-Jade B-stained cells emit a typical green color with excitation peak at 480 nm and emission peak around 525 nm.

Cell Culture and Small Interfering RNA (siRNA) Transfection

We assessed the excitotoxic effect of 200 μM KA on Neuro2A (N2A) cells with low levels of PrPc expression by using siRNA transfection. PrPc siRNA used for prion silencing and the scramble siRNA sequence used as a control were obtained from (Daude et al., 2003) and were synthesized by Darmacon (Lafayette, LA). N2A cells were grown at 37°C, 5.5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, 10% FBS, and antibiotics. For siRNA transfection, cells were plated at 1×10^5 cells/well in a 24-well plate and transfected the next day using Lipofectamine 2000, following the manufacturer's instructions (Invitrogen-Life Technologies). Briefly, 1 μl Lipofectamine 2000 was mixed with siRNAs (400 nM) in 50 μl of Opti-MEM medium and after 30 min at room temperature it was added to N2A cells for 4 hr. The decrease in PrPc expression after treatment was checked by Western blot analysis. KA-treatments were carried out on serum-deprived cells 48 hr after siRNA transfection. Afterward, KA-treated cells were incubated with 1 μg/ml PI for 1 hr, then fixed in 1% paraformaldehyde in 0.1 M PBS and counterstained with DAPI (1 μM) to corroborate equal cell densities. For quantification, the number of PI-labeled cells was counted in a 500-μm^2 square using a 40× oil immersion objective. In parallel experiments, total mRNA was obtained from siRNA (*Prnp* and scrambled) treated cultures and processed to determine the expression level of KA receptor subunits by real-time RT-PCR.

Semi-Quantitative RT-PCR of AMPA/KA Receptors

Two micrograms of total RNA obtained from *Prnp* −/− and wild-type mice through purification with Trizol (Invitrogen-Life Technologies) were retrotranscribed using AMV

reverse transcriptase (New England Biolabs, Herts, UK). Both steps were carried out following the manufacturer's instructions, except that we used our antisense oligonucleotides for reverse transcription (instead of olgo-dT). Three micrograms of cDNA (corresponding to $\frac{1}{20}$ of the total RNA retrotranscribed) were amplified with Taq polymerase (Amersham Biosciences, Buckinghamshire, UK) in a mixture containing dNTPs, Taq buffer, 1.25 mM $MgCl_2$ and oligonucleotides to a final volume of 25 μl. The oligonucleotide sequences were the same as in the study by Paarmann et al. (2000) in all cases, except for glyceraldehyde-3-phosphate dehydrogenase *gapdh* cDNA amplification, the sequences of which were the same as in the study by Burgaya and Girault (1996). A total of 7.4 μCi of ^{33}P-dATP (Amersham Biosciences) was added to each reaction as a tracer.

Each amplification cycle consisted of 30 sec denaturation at 94°C, 30 sec of annealing at 60°C and 35 sec elongation at 72°C. A single, previous step of 2 min at 94°C and a final elongation of 7 min at 72°C were used for optimal resolution. Quantitative amplification and finest viewing of the radiolabeled bands was achieved after 15 cycles for the multi-copy gene *gapdh*, and 20 or 25 cycles for the glutamate receptor subunit cDNAs. Five microliters of 6× loading buffer (30% glycerol-bromophenol blue) was added to the mixture before PAGE electrophoresis in 4% acrylamide/bis-acrylamide gels, run in 1× TAE buffer. Gels were fixed for 30 min in 7% methanol–5% acetic acid and vacuum-dried over Whatman paper for 2 hr at 60°C before exposure to sensitive plates for 1–18 hr, and analyzed in a Typhoon 8600 scanner (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Molecular Dynamics).

Real-Time PCR

Quantitative PCR of was carried out using an ABI PRISM 7700 sequence detection system equipment and power Sybr green master mix (Applied Biosystems). Reaction volumes of 12.5 μl were used with 0.5 μM primers. Specific primers were taken from a database (http://pga.mgh.harvard.edu/primerbank/) based on the published sequences of mouse *gapdh* (NM_008084) (primers: forward, 5′-AGGTCGGTGTGAACGGATTTG-3′; reverse, 5′-TGTAGACCATGTAGTTGAGGTCA-3′) as referenced together with glutamate receptors GluR6, AF245444 and GluR7, D10054 (GluR6, primers: forward, 5′-ATCGGATATTCGCAAGGAACC-3′; reverse, 5′-CCATAGGGCCAGATTCCACA-3′ and GluR7, primers: forward, 5′-AGGTCCTAATGTCACTGACTCTC-3′; reverse, 5′-GCCATAAAGGGTCCTATCAGAC-3′). Amplification conditions consisted of 2 sec denaturation at 95°C, 15 sec of annealing at 60°C, and 1 min elongation at 60°C for 40 cycles. The results were normalized by the expression levels of the housekeeping gene, *gapdh*, which was quantified simultaneously with the target gene. We added 2 μl of the serially diluted cDNA prepared from tissue to this mixture. A melting point analysis was carried out to improve the sensitivity and specificity of amplification reactions detected with the Sybr Green I dye. Data were analyzed by SDS 1.9.1 Software (Applied Biosystems) following the $2^{-\Delta\Delta C_T}$ method of Applied Biosystems published by Livak and Schmitgen (2001).

Western Blotting Techniques

Tissue samples from the hippocampi of KA-treated mice were homogenized (10% w/v) in ice-cold Tris-saline buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.5% (w/v) Triton X-100, 0.5% (w/v) Nonidet P-40 (Igepal; Sigma), and 1× cocktail of protease and phosphatase inhibitors, using a motor-driven glass-Teflon homogenizer in ice. After protein quantification, tissue extracts (20 μg) were boiled in Laemmli sample buffer at 100°C for 5 min, followed by 6–10% SDS-PAGE electrophoresis, electro transferred to nitrocellulose membranes for 6 hr at 4°C and processed for immunoblotting using primary antibodies and the ECL-plus kit (Amersham-Pharmacia Biotech). In our experiments, each nitrocellulose membrane was used for detecting both phosphorylated and total kinase levels.

RESULTS

KA Sensitivity, Seizure Behavior, and Mortality in *Prnp* −/− and Wild-Type Mice

The thresholds for onset and seizure behavior in response to identical intraperitoneal KA injections differed greatly daggers, between *Prnp* −/−, heterozygote, and wild-type mice (daggers, Table I). Two *Prnp* −/− mice died whereas only one wild-type mouse died during the experiments. Only two wild-type mice and no heterozygote mice showed small (Grade III, hyperactivity) seizures after KA injection. In contrast, PrPc-deficient mice showed an increased number of seizures (ranking from 13–19) with profound seizure impact behavior (Grade V–VII). In addition, they maintained Grade V–VI seizure for 3–6 hr whereas wild-type and heterozygote mice displayed only grade III seizure for 3 hr. These data corroborate the results of Walz et al. (1999) reviewed in Walz et al. (2002), and indicate particular sensitivity of *Prnp* −/− mice to KA. Walz et al. (1999) reported that 50% of prion mutant mice showed repetitive seizures whereas no wild-type exhibited observable seizures. In addition, our experiments extend their observations to heterozygote mice, which displayed similar behavior to that of wild-type animals. After 7–8 hr, wild-type and heterozygote mice showed normal behavior and the symptoms of *Prnp* −/− KA-treated mice decreased gradually to a level of hypoactivity and immobility that was corroborated 12 hr after treatment. This hypoactivity was maintained until the histologic or biochemical processing of the mice.

Increased Seizure-Related Histopathology in *Prnp* −/− Mice

To determine whether the severity of seizure observed in *Prnp* −/− correlates with the pattern of neuronal loss and reactive glial changes after KA injection as described in the literature, we carried out several histochemical and immunohistochemical analyses (Figs. 1–3).

Sections from adult animals were labeled with Fluoro-Jade B stain, a marker for neuronal degeneration (Schmued and Hopkins, 2000) (Fig. 1). Two days after

TABLE I. Effects of KA-Induced Status Epilepticus and Death on *Prnp* −/−, Heterozygotes and Wild-Type Mice

Genotype	Onset (min)	No. of seizures	Behavioral stages	Prioritary stage (min)
Prnp −/−	40	14	I–VI	V–VI (6:20 hr)
Prnp −/−	118	13	I–VI	V–VI (5 hr)
Prnp −/−	103	19	I–VI	V–VI (6 hr)
Prnp −/−	120	15	I–VI	V–VI (4 hr)
Prnp −/−	100	13	I–VI	V–VI (5 hr)
Prnp −/−	13	Continue	I–VII	†
Prnp −/−	27	Continue	I–VII	†
Prnp −/−	160	15	I–VI	V–VI (3 hr)
Prnp +/−	300	1	I–II	III (3 hr)
Prnp +/−	0	0	I–III	III (5 hr)
Prnp +/−	0	0	I–III	III (3 hr)
Prnp +/+	61	1	I–VI	III–IV (3 hr)
Prnp +/+	0	0	I–III	III (5 hr)
Prnp +/+	31	Continue	I–VII	†
Prnp +/+	45	1	I–VII	III (2 hr)
Prnp +/+	300	1	I–VI	III (5 hr)
Prnp +/+	0	0	I–III	III (6 hr)

the last KA injection, *Prnp* −/− mice displayed numerous cells with Fluoro-Jade B fluorescence in the hippocampus, amygdala, and piriform cortex. In the hippocampus, numerous labeled cells were located in the pyramidal layer of the CA1 and CA3 regions (Fig. 1A,B,E–G). In contrast, heterozygote and wild-type C57BL6J mice displayed no fluorescence in cells in these regions (Fig. 1C,D,H–J). Fluorescence labeling in pyramidal cells extended from the neuronal perikaryon to apical dendrites often displaying disrupted or condensed chromatin after DAPI counterstaining (Fig. 1E–G).

In addition, increased c-fos immunoreactivity in the same regions as above was observed in KA-treated *Prnp* −/− in contrast to controls. Similar c-fos immunoreactivity distribution after KA injections was also reported by Popovici et al. (1990) in KA-treated rats. We observed particularly marked immunoreactivity in *Prnp* −/− sections in the pyramidal layer of the CA1-3 (Fig. 2). Numerous nuclei were labeled in *Prnp* −/− mice in contrast to controls, where only a diffuse labeling in pyramidal neurons was observed (Fig. 2A–D). Moreover, it is well known that the activation of glial cells begins 2–3 days after KA treatment (Jorgensen et al., 1993). Indeed, abundant GFAP-positive cells displaying hypertrophy and thick processes were observed in the neurodegenerative areas of the hippocampus in *Prnp* −/− mice in contrast to wild-type mice (Fig. 2E,F). The present results indicate that KA-induced seizure in prion mutant mice correlates with the histologic descriptions of cell death and reactive gliosis in the hippocampal region.

Increased Activation of Extracellular-Related Kinases (ERK 1-2), P38 Stress-Associated Kinase, and c-jun N-Terminal Kinase in the Hippocampi of KA-Treated *Prnp* −/− Mice

Protein kinase-mediated signalling cascades constitute the major route by which neurons and glial cells respond to their extracellular environment. Mitogenic or stress stimulation of the ERK1-2 pathway modulates the activity of many transcription factors, leading to biologic responses such as proliferation and differentiation (Volmat and Pouyssegur, 2001). In addition, the p38/SAPK2 pathways are activated strongly by stress stimuli (Irving and Bamford, 2002). In addition, c-jun N-terminal kinase (JNK) activation has also been associated with cell death. There is a growing body of evidence showing that these kinase signalling pathways are activated by a variety of injury stimuli including focal cerebral ischemia (Irving and Bamford, 2002) or glutamate/KA excitotoxicity (Mao et al., 2004). In our experiments, KA-treated *Prnp*−/− mice displayed relevant activation of both ERK1-2 and P38 kinases in Western blot of hippocampal extracts compared to KA-treated controls (Fig. 3A). Densitometric quantification showed a 2.4-fold increase in ERK1-2 activation and a 1.8-fold activation of P38 in *Prnp* −/− mice (Fig. 3B). Histologic correlation using double immunofluorescence showed that cells in prion mutant mice expressing activated ERK1-2 after KA-injection corresponded to reactive astroglia (GFAP-positive) (Fig. 3C, D,G,L–N). In addition, pP38-immunoreacted sections of KA-treated *Prnp* −/− mice showed a relevant increase in the labeling of mossy fibre projections, a decrease in neuronal pP38 labeling in pyramidal neurons, and increased P38 in reactive astrocytes compared to KA-treated controls (Fig. 3E,F, H,I), which showed similar labeling to that detected in untreated mice. In addition, pJNK staining was relevant after KA-treatment in mossy fibers (not shown) and pyramidal cells of CA1-3 in *Prnp* −/− mice compared to controls (Fig. 3J,K). These results corroborate the notion that the sensitivity of *Prnp* −/− mice to KA-induced seizure correlates with the increased activation of ERK1-2, P38 in glial cells and JNK in neurons of brain regions susceptible of the lethal effects of the status epilepticus.

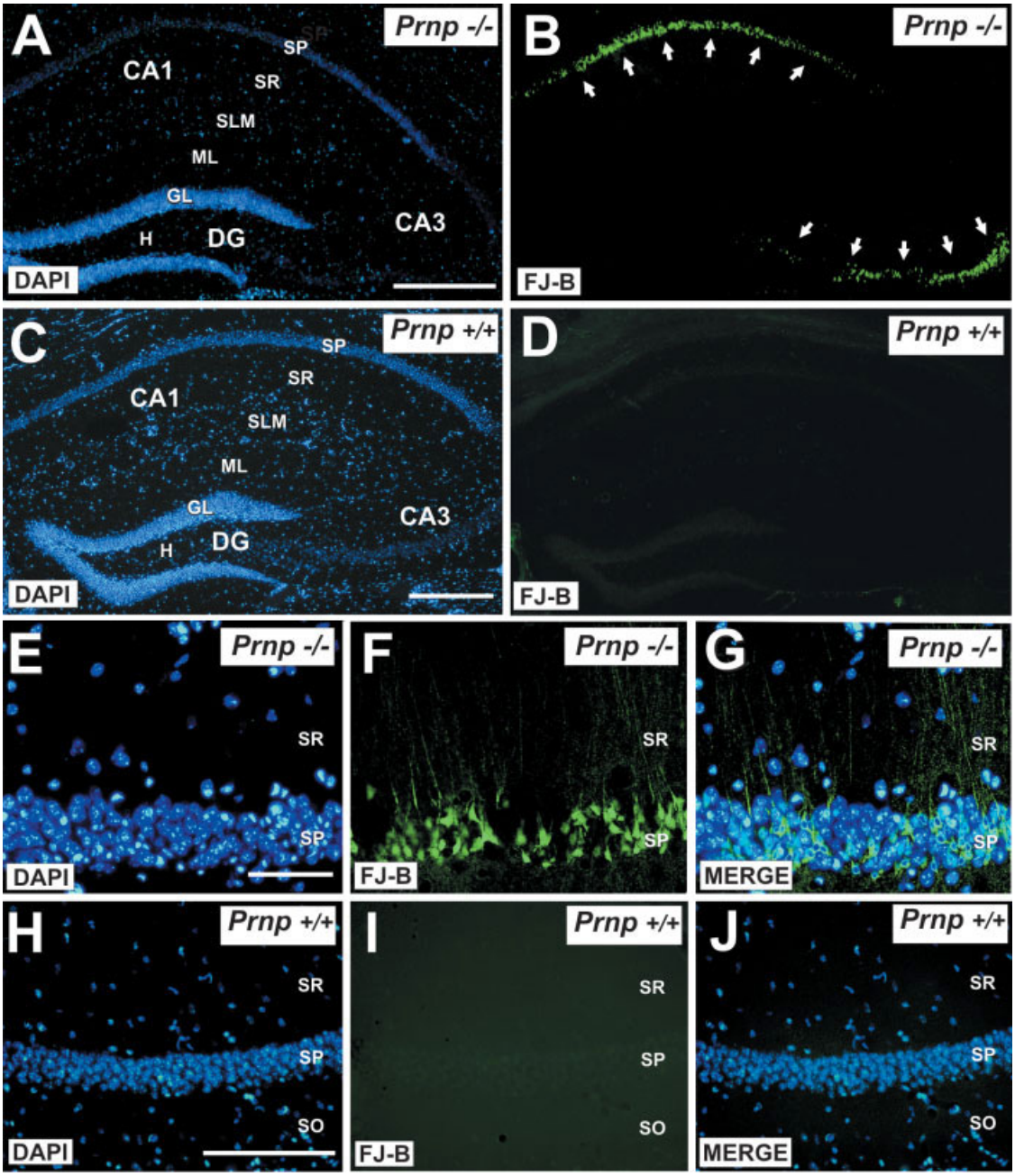

Fig. 1. Low power photomicrographs illustrating the pattern of Fluoro-Jade B labeling (**B,C,F,G,I,J**) and DAPI counterstaining (**A,C,E,H**) in the hippocampi of PrPc-deficient (A,B,E–G) and control (C,**D**,H–J) mice after treatment with KA. Fluoro-Jade B labeled cells were also observed in *Prnp* −/− mice especially in the CA1-3 region (B). Higher magnification shows that Fluoro-Jade B labels the perikaryon and the apical dendrites of dying pyramidal cells (F). Cells labeled with Fluoro Jade-B displayed condensed chromatin, as ascertained by DAPI staining (E,G). Scale bars: (A,C) 500 μm pertains to (B) and (D) respectively; (E,H), 100 μm pertains to (F,G) and (I,J) respectively. *Abbreviations:* CA1-3, hippocampal fields; DG, dentate gyrus; GL, granular layer; H, hilus; ML, molecular layer; SLM; stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale, SR, stratum radiatum.

KA-Induced Seizure Responses in the Hippocampi of *Prnp* −/− Mice Are Cell-Specific

Hippocampal damage induced by administration of KA may be triggered not only by direct effect of this compound on pyramidal neurons, but also through the hyperactivity of the excitatory afferent pathway, i.e., the entorhino-hippocampal (EH) connection (Sperk, 1994). To ascertain whether neurotoxic differences are cell-specific or related to increased excitability of the activated EH connection, we developed a parallel study in isolated

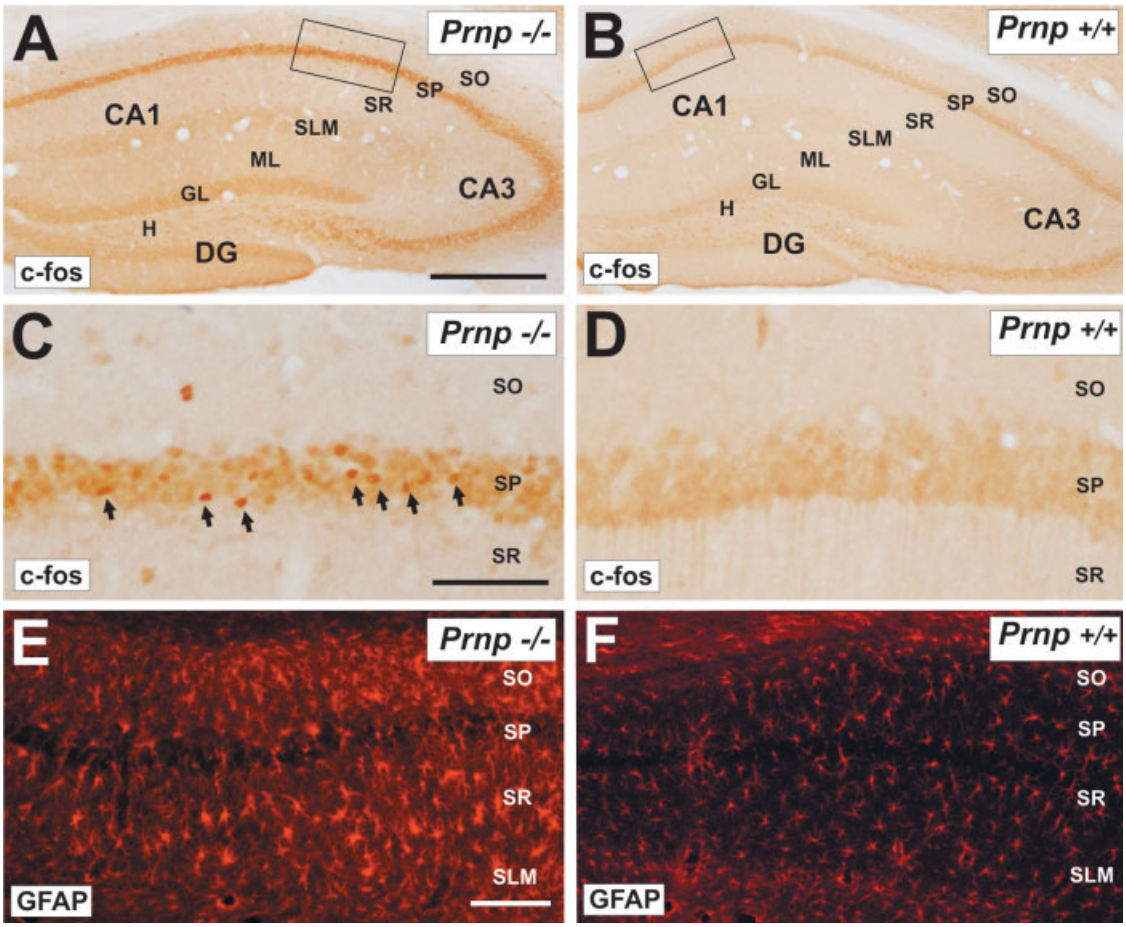

Fig. 2. **A,B:** Low power view of the hippocampal region illustrating the distribution of c-fos immunoreactivity in the hippocampi of PrPc-deficient and control mice after intraperitoneal KA injections. **C,D:** are a higher magnification of the squares shown in (A,B) respectively. PrPc-deficient hippocampi showed increased c-fos nuclear staining in pyramidal neurons (arrows in C). **E,F:** Photomicrographs of the CA1 showing the pattern of GFAP immunostaining in mutant mice (E) and control (F) after KA treatment. GFAP immunostaining strongly increased in the CA1. Scale bars: (A), 500 μm pertains to (B); (C,E), 100 μm pertains to (D) and (F) respectively. *Abbreviations:* CA1-3, hippocampal fields; DG, dentate gyrus; GL, granular layer; H, hilus; ML, molecular layer; SLM; stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale, SR, stratum radiatum. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hippocampi in organotypic slice cultures (Fig. 4). *Prnp* −/− and wild-type hippocampal slices were treated with KA, 0.1 M PBS, or glutamate. The levels of PI uptake were then measured in the pyramidal layer of the hippocampus (Fig. 4A–D). *Prnp* −/− slices showed a 2.6-fold increase in PI-labeled cells compared to controls, the fluorescence levels of glutamate and PBS being considered the maximum and minimum levels for quantification, respectively (Fig. 4E). These data suggest that the increased cell death observed in the hippocampal cells corresponds to cell-specific differences in the response to KA treatment not to increased or modified extra-hippocampal afferent stimulation. In a parallel experiment we explored the participation of NMDA receptors in the KA-induced cell death in *Prnp* −/− slices by using several concentrations of the non-competitive antagonist MK-801. Indeed, MK-801-treated cultures displayed a significant reduction of 15% (10 μM MK-801) and 40% (30 μM of MK-801) of cell death in *Prnp* −/− slices after KA-treatment. These observations indicate the participation of NMDA receptors in the KA-induced cell death in *Prnp* −/− mice organotypic cultures.

Silencing of the *Prnp* Gene Induces KA-Dependent Cell Death in Neuroblastoma Cells

We explored whether the specific silencing of the *Prnp* gene induces sensitivity to KA in normal PrPc-expressing cells. First, we incubated N2A cells with siRNA plus a plasmid encoding eGFP (pcDNA3-eGFP) to explore transfection efficiency using Lipofectamine 2000 (Fig. 5). Microscopic observation showed that 70–80% of N2A cells were transfected (Fig. 5). The decrease in PrPc expression in siRNA (*Prnp*)-transfected cultures was later corrobo-

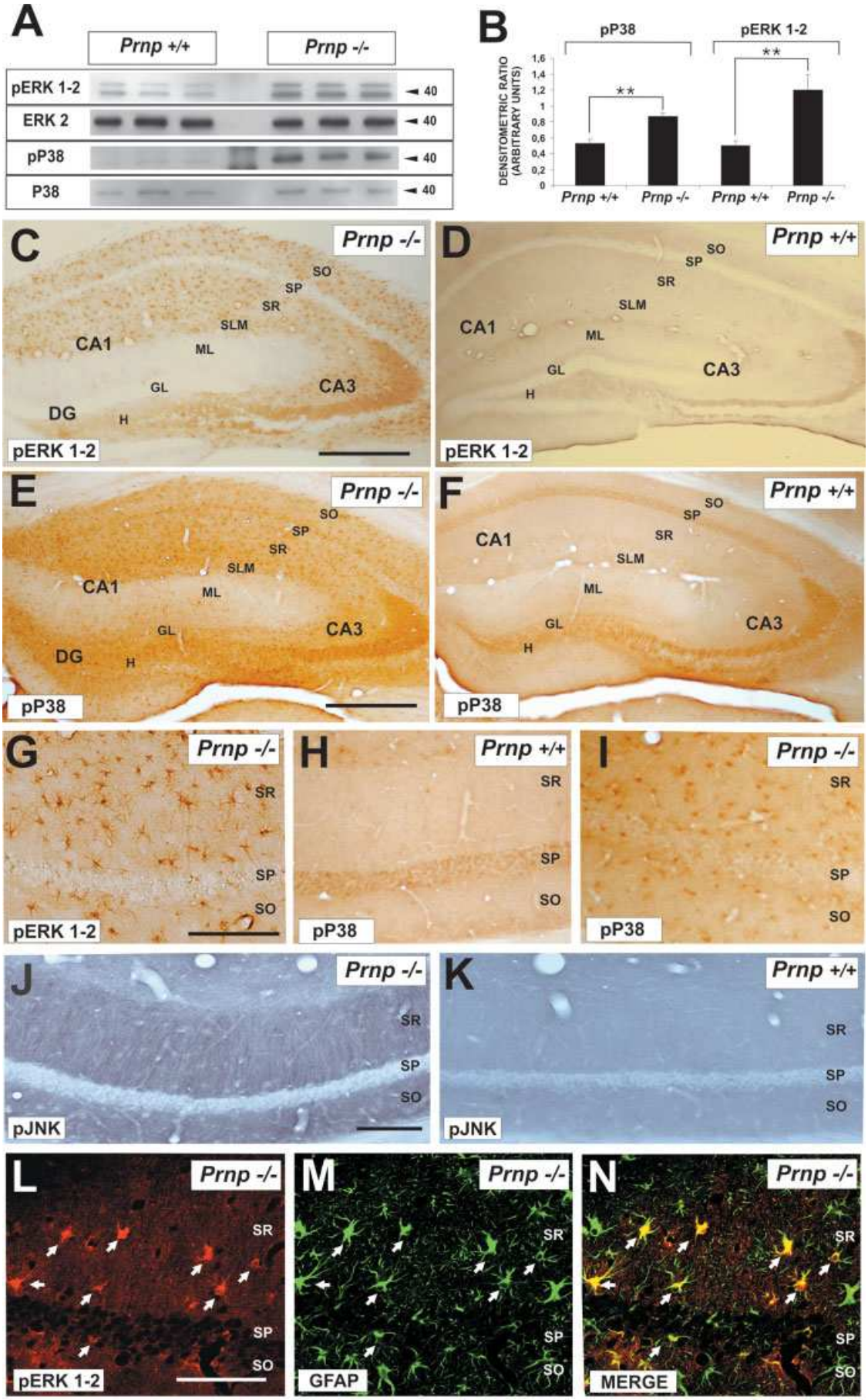
A
Prnp +/+
Prnp -/-
pERK 1-2
ERK 2
pP38
P38
40
B
pP38
pERK 1-2
DENSITOMETRIC RATIO (ARBITRARY UNITS)
Prnp +/+
Prnp -/-
C
D
E
F
SO
SP
SR
SLM
ML
GL
CA1
CA3
DG
H
pERK 1-2
pP38
G
H
I
J
K
pJNK
L
M
N
GFAP
MERGE

Figure 3.

rated by Western blot (Fig. 5B). Furthermore, siRNA(*Prnp*)-, siRNA(scrambled)-, and Lipofectamine-transfected N2A cells were treated with 200 μM of KA and further incubated with PI (Fig. 5C–G). We observed a 4.8-fold increase in the number of PI-labeled cells between siRNA(*Prnp*)- and siRNA(scrambled) cells treated with KA (Fig. 5C–G). These data indicate that the specific silencing of PrPc increased sensitivity to KA in cultured N2A cells, which supports the notion of a protective role of PrPc.

Enhanced Susceptibility to KA-Induced Seizures in PrPc-Deficient Mice Correlates With Differential Expression of GluR6-7 KA Receptor Subunits in the Hippocampus

Ionotropic glutamatergic neurotransmission in the CNS is mediated by NMDA and AMPA/KA receptors. *Prnp* −/− mice present a diminished hyperlocomotor response to MK-801 treatment compared to wild-type animals, indicating that lack of PrPc leads to a functional alteration of the glutamatergic system mediated by NMDA receptors (Coitinho et al., 2002). In addition, mutant mice show increased expression of NMDA receptor subunits NR2A and NR2B (Maglio et al., 2004). However, literature provides no data concerning differential expression of AMPA/KA receptors in PrPc-deficient mice. We explored whether the sensitivity of prion mutant mice to KA-induced seizure observed in our experiments correlates with differential expression of AMPA/KA receptor subunits in hippocampal neurons (Fig. 6). First, coronal sections from mutant and control mice were histologically processed to ascertain putative differences in the pattern of expression of AMPA/KA receptor subunits in the hippocampal formation. No relevant differences were observed in the immunocytochemical distribution pattern of the diverse AMPA/KA receptor subunits in the hippocampi of mutant mice compared to controls (not shown). Furthermore, to better determine putative differences in subunit expression, we carried out a radioactive semi-quantitative RT-PCR study (Fig. 6A,B). We confirmed that GluR1, GluR2, GluR3, GluR4, and GluR5, as well as KA1 and KA2 mRNA levels in *Prnp* −/− mice remained similar to wild-type hippocampus (Fig. 6B). However, GluR6 and GluR7 mRNA levels were statistically increased in *Prnp* −/− mice compared to controls (Fig. 6A,B). This was corroborated using Sybr green real-time RT-PCR (Fig. 6C–E) and statistical analysis. Gel electrophoresis and melting point analysis were carried out to determine the specificity of the amplified products (Fig. 6C,D). Quantification of the data showed a 2.33-fold increase and a 2.06-fold increase in the mRNA levels of GluR6 and GluR7, respectively in *Prnp* −/− compared to wild-type (Fig. 6E). These data indicate the increased expression of GluR6 and GluR7 KA receptor subunits in the hippocampi of prion mutant mice, and suggest their participation in the increased susceptibility to KA in these animals. The presence of GluR6-7 KA-receptor subunits in susceptible cells (hippocampal pyramidal cells) was corroborated by immunohistochemistry in *Prnp* −/− mice (Fig. 6F). Last, we determined that neuroblastoma cells treated with siRNA-*Prnp* showed increased GluR6 and GluR7 mRNA (Fig. 6G). The present data point to a putative relation between PrPc and GluR6 and GluR7 expression, similar to the relation reported previously for some NMDA receptor subunits (Maglio et al., 2004)

DISCUSSION

In recent years considerable research has addressed the functions of PrPc (Martins et al., 2002; Sakudo et al., 2006). Emerging data indicate that PrPc is crucial in neuroprotection. This GPI-anchored protein protects neurons against oxidative stress (Brown et al., 2001) or bax-mediated apoptosis (Bounhar et al., 2001), probably through its interaction with the stress-inducible protein 1 and activation of the PKA/cAMP signalling pathway (Chiarini et al., 2002; Zanata et al., 2002). In addition, recent studies also report parallel functions for PrPc. For example, this protein promotes neuritogenesis by binding to laminin (Graner et al., 2000) or neural cell adhesion molecule (NCAM) (Santuccione et al., 2005). With respect to synaptic transmission, although mutant mice were initially described without clear behavioral deficits (Bueler et al., 1992), further studies proposed the involvement of PrPc in normal synaptic function because *Prnp* −/− mice displayed impaired LTP (Collinge et al., 1994). In addition, disrupted Ca^{2+}-activated K^{+} currents (Colling et al., 1996) and axon abnormalities have been

Fig. 3. **A:** Immunoblot of pERK1-2, ERK, pP38 and P38 in the hippocampi of PrPc-mutant mice and control after KA-induced seizure. Samples (50 μg) from hippocampi of the ages indicated were analyzed. The molecular weight standards are shown on the right. **B:** Histogram illustrating the densitometric analysis of the immunoblots shown in (A). Quantitative results from three independent experiments are illustrated. Values are represented as mean ± SD. *Statistical differences between columns ($P < 0.05$; ANOVA) **C–F:** Low power photomicrograph of the hippocampus showing the distribution of pERK1-2 (C,D) and pP38 (E,F) in *Prnp* −/− and control mice 72 hr after KA administration. Note the overall increase in both activated kinases in the CA1-3 of the hippocampus and especially the increase in mossy fibre labeling in PrPc-deficient mice. **G–K:** High magnification of the CA1 immunostained against pERK1-2 (G), pP38 (H,I), and pJNK (J,K). Note the increase in pERK1-2 and pP38 in reactive astroglia in *Prnp* −/− mice. In contrast, neuronal labeling of P38 decreased in the pyramidal layer, whereas pJNK increased after treatment with KA. **L–N:** Examples of double-labeled astrocytes (GFAP + pERK1-2) (arrows) in the CA1 of *Prnp* −/− mice 72 hr after the final KA injection. Scale bars: (C,E), 500 μm pertains to (D) and (F), respectively; (G,J), 100 μm pertains to (H,I) and (K), respectively; (L), 100 μm pertains to (M,N). *Abbreviations:* CA1-3, hippocampal fields; DG, dentate gyrus; GL, granular layer; H, hilus; ML, molecular layer; SLM; stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale, SR, stratum radiatum.

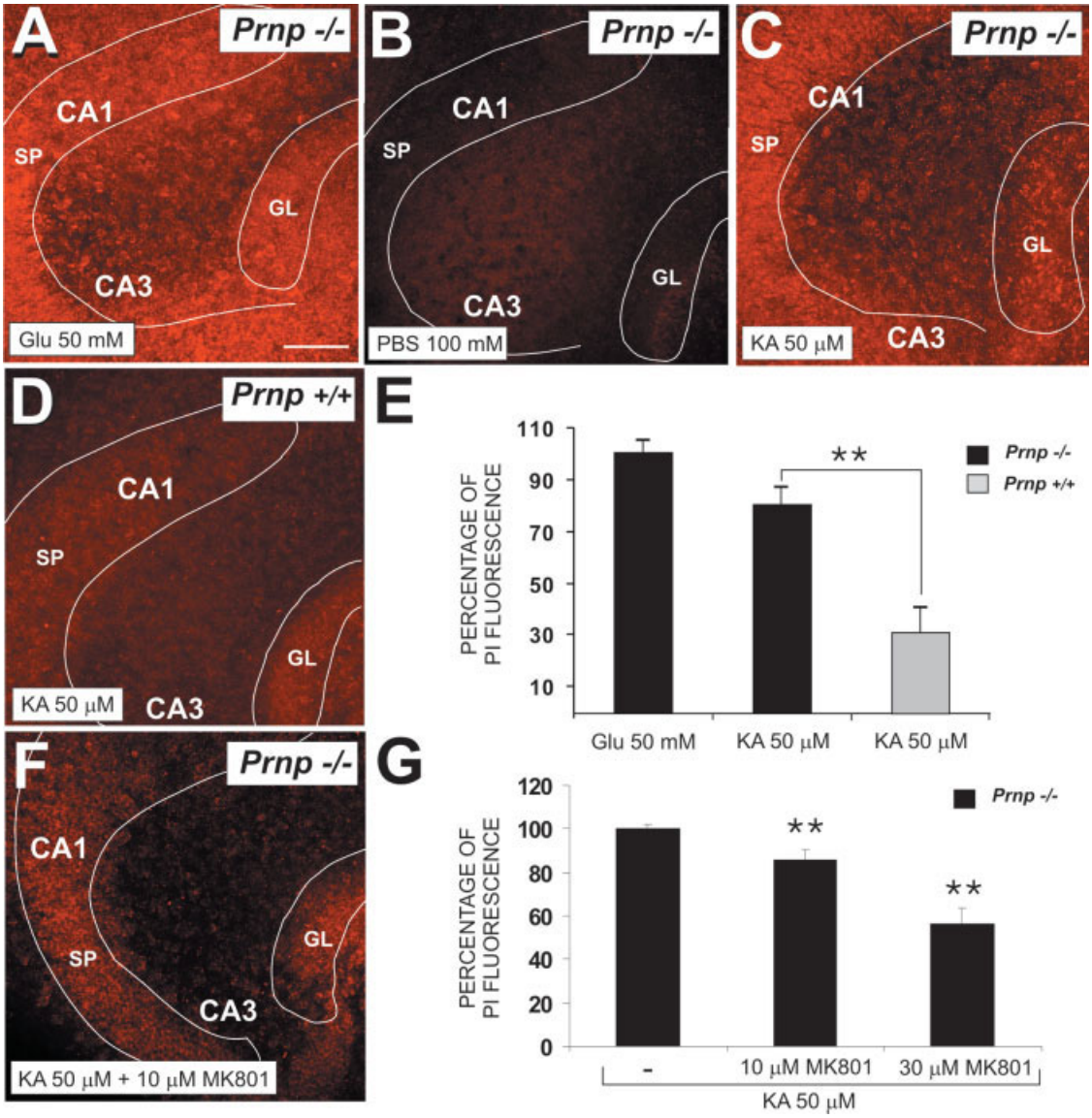

Fig. 4. **A–D:** Fluorescence images of organotypic hippocampal slice cultures from PrPc-deficient (A–C) and wild-type (**D**) mice incubated with 50 mM glutamate (A), PBS 100 mM (B) or 50 μM KA (1 μg/ml) (C,D) and incubated with propidium iodide (PI). Note the relevant PI-incorporation in dying cells in *Prnp* −/− cultures compared to controls **E:** Histogram illustrating quantitative results of (A–D). **F:** Fluorescence images of hippocampal organotypic slice culture treated with 10 μM MK-801 and 50 μM KA. Note the presence of PI-labeled cells. **G:** Histogram illustrating quantitative results of the MK-801 experiments. Note the decrease in the number of PI-labeled cells specially with 30 μM MK-801. Histograms in (E) and (G) represent the mean ± SD of three independent experiments. *Statistical differences between columns ($P < 0.05$; ANOVA). Scale bars: (A), 200 μm pertains to (B–D,F). *Abbreviations:* CA1-3, hippocampal fields; DG, dentate gyrus; GL, granular layer; H, hilus; ML, molecular layer; SLM; stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale, SR, stratum radiatum. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

reported in mutant mice (Colling et al., 1997). However, these data were controversial because normal neuronal excitability and synaptic transmission was described in the hippocampus of *Prnp* −/− mice (Lledo et al., 1996). Despite these discrepancies, it was shown recently that PrPc-deficient mice display facilitated glutamatergic transmission with a lower threshold for generating LTP compared to wild-type animals and paralleled by an increased expression of the NMDA receptor subunits NR2A and NR2B in the hippocampi of mutant mice (Maglio et al., 2004). In addition, the same authors showed recently increased LTP in older mutant mice compared to controls (Maglio et al., 2006). These latter data indicate that PrPc is involved in glutamatergic neurotransmission and neuron homeostasis. In this regard, Walz et al. (1999) described increased sensitivity to KA-mediated seizures in PrPc-deficient mice, which was further corroborated in vitro in cell cultures (Brown et al., 2002). To date, how-

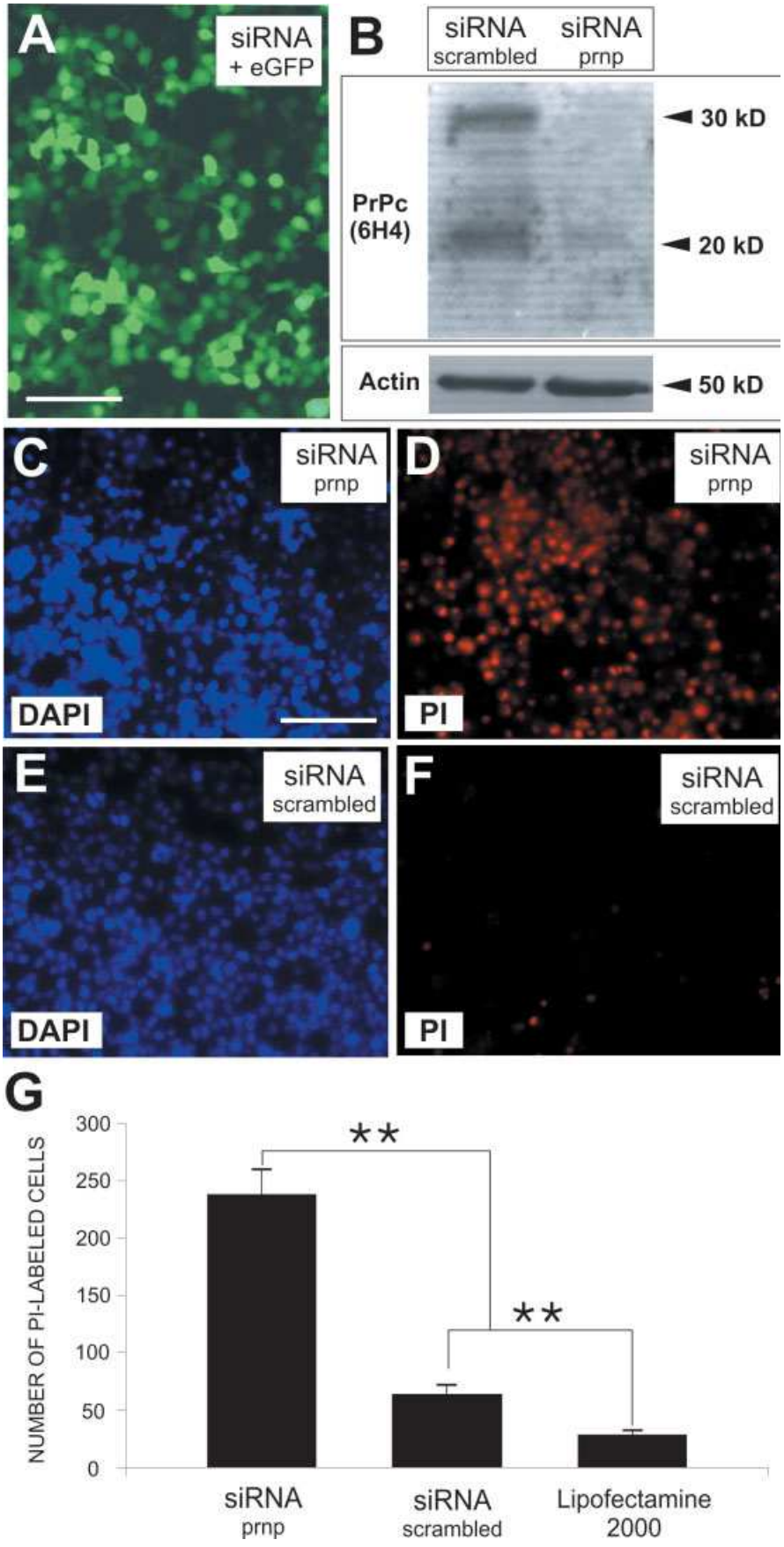

Fig. 5. **A:** Example of double transfected N2A cells with siRNA(*Prnp*) and eGFP- expressing plasmid. Note that most N2A cells are labeled. **B:** Western blot corroboration of the decrease in PrPc protein in N2A-transfected cell protein extracts after the interference experiment. **C–F:** Low power image of N2A cultures transfected with siRNA(*Prnp*) (C,D) or siRNA(scrambled) (E,F) and treated with KA. Note that siRNA(*Prnp*)-transfected cells showed increased PI-uptake (C,D) in contrast to siRNA(scrambled)-N2A-transfected cells (E,F). **G:** Histograms represent the mean ± SD of three independent experiments. *Statistical differences between columns ($P < 0.05$; ANOVA). Scale bars: (A), 75 μm; (C), 100 μm pertains to (D,E).

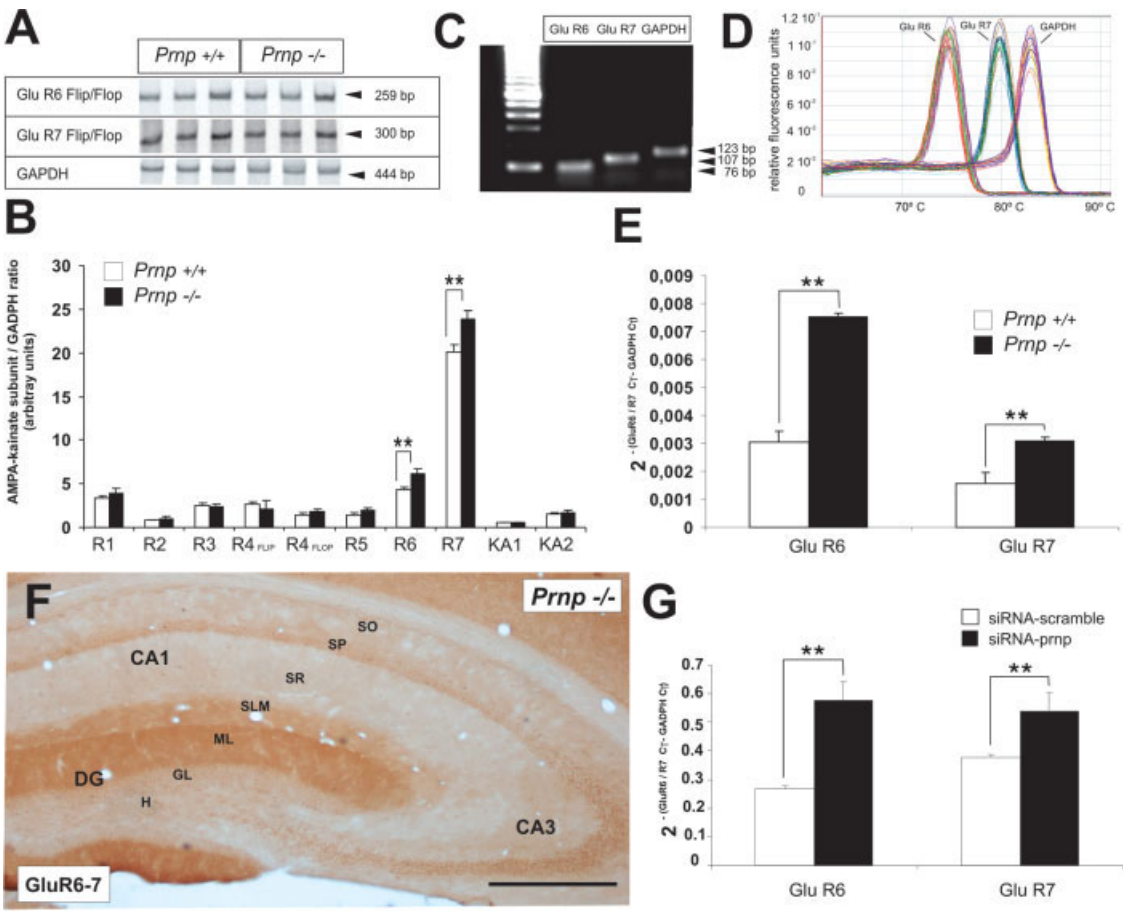

Fig. 6. RNA expression of AMPA/KA receptors subunits in *Prnp* −/− and wild-type hippocampal tissue and after silencing of PrPc in N2A cells. **A:** RT-PCR-amplified cDNAs of the GluR6 and GluR7 subunits and GAPDH from hippocampal extracts were mixed in a single tube and electrophoretically separated in 4% acrylamide gels. The amplified bands were identified on the basis of their size (right site, e.g., 444 bp for GAPDH cDNA) and developed by Typhoon 8600 scanning. **B:** Densitometric quantification of the AMPA/KA subunit/GAPDH ratio of RNA expression from hippocampal extracts in *Prnp* −/− (black bars) and wild-type (open bars) mice. *Statistical differences between columns ($P < 0.05$; ANOVA). **C,D:** Agarose gel (C) and parallel melting point experiment of the amplified products using the Sbgr real-time RT-PCR primers for GluR6, GluR7, and GAPDH. The size of the amplified bands are identified on the right side of (C). Note the presence of a single amplification product in (C) and the three independent curves in the melting point experiment in (D), which indicate the efficiency of the amplified products using the primers described. **E:** Histogram illustrating the quantitative results of the real-time RT-PCR experiment of GluR6 and GluR7 mRNA levels in *Prnp* −/− (black bars) and wild-type (open bars) hippocampi. **F:** Low power magnification illustrating the distribution of GluR6-7 in *Prnp* −/− mouse hippocampus. Note the relevant labeling of the pyramidal layer, and the dentate molecular layer and the slm of the CA1-3. **G:** Histogram illustrating the quantitative results of the real-time RT-PCR experiment of GluR6 and GluR7 mRNA levels in N2A cells after transfection with siRNA(*Prnp*) (black bars) or siRNA(scramble) (open bars). Note the increased expression of GluR6 and GluR7 after siRNA(*Prnp*) transfection. Histograms in (E) and (G) represent the mean ± SD of three independent experiments. *Statistical differences between columns ($P < 0.05$; ANOVA). Scale bar: (C), 500 μm; (D), 200 μm. *Abbreviations:* CA1-3, hippocampal fields; DG, dentate gyrus; GL, granular layer; H, hilus; ML, molecular layer; SLM; stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale, SR, stratum radiatum. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ever, no detailed histologic correlation of KA-induced seizures or AMPA-KA receptor expression analysis in these mutant mice is available.

Our results corroborate that young adult *Prnp* −/− mice show increased sensitivity to KA-induced seizures compared to wild-type mice under identical KA treatments. This seizure sensitivity correlates with the well-described pattern of neuronal degeneration in several brain regions susceptible to KA (e.g., hippocampal region, amygdala, or piriform cortex). We also show that neuronal death correlates with enhanced activation of the stress-associated kinases P38 and ERK1-2 in the KA-treated hippocampi of PrPc-deficient mice compared to wild-type mice. Epileptic status rapidly induces the activation of both kinases in the CA1-3 hippocampal fields (Jeon et al., 2000; Ferrer et al., 2002). Interestingly, we corroborate that the expression of these two kinases in *Prnp* −/− mice occurs in reactive astrocytes in the hippo-

campal CA1-3, as reported in control mice in other studies (Che et al., 2001; Ferrer et al., 2002). In addition, a decrease in activated p38 was observed in dying pyramidal cells in the CA1-3 region, which paralleled increased JNK activation in these neurons.

Many studies have reported that the absence of PrPc leads to a particular neuronal phenotype that is sensitive to oxidative stress (Brown et al., 2002; Sakudo et al., 2006). PrPc-deficient mice show increased basal levels of phospho-ERK1-2 as well as other intracellular proteins (e.g., p53) or anti-apoptotic factors (e.g., bcl-2) (Brown et al., 2002). Increased activation levels of P38 and ERK1-2 are involved in KA-induced seizure preconditioning and ischemic tolerance (Gonzalez-Zulueta et al., 2000; Jiang et al., 2005). This increased expression of ERK1-2 in healthy untreated PrPc-deficient mice may explain the normal phenotype of *Prnp* −/− cells, because sublethal activation of ERK1-2 and p38 might protect neurons against the basal stress levels observed in PrPc-deficient neurons. Under conditions of increased stress, however, such as those reported in the present study (KA-injection), these palliative mechanisms cannot further support neuron survival.

To date, five subunits of KA receptors have been identified, GluR5, GluR6, GluR7, KA1, and KA2 (Hollmann and Heinemann, 1994; Lerma, 2006). GluR5-7 subunits display lower affinity for KA than KA1-2 (Hollmann and Heinemann, 1994). However, GluR5-7 form homomeric and heteromeric receptor channels, in contrast to KA1-2, which assemble only heteromerically. We have shown the expression of GluR6-7 in pyramidal neurons of the hippocampal CA1-3. In addition, our RT-PCR experiments show that endogenous mRNA levels of GluR6 and GluR7 KA receptor subunits are increased in the hippocampi of PrPc-deficient mice compared to controls. In addition, the silencing of PrPc production in neuroblastoma cells by siRNA leads to an increased expression of GluR6 and GluR7. Several studies report the relevance of GluR6 in susceptibility to KA. For example, lower levels of susceptibility to this compound have been described in mice devoid of GluR6 (Mulle et al., 1998). In addition, the overexpression of GluR6 in rat hippocampal slices induces seizures and spontaneous bursting (Telfeian et al., 2000) whereas treatment with GluR6 antisense oligonucleotides or specific peptides produces neuroprotective effects in the ischemic hippocampus (Pei et al., 2005). These findings indicate that the susceptibility to KA observed in *Prnp* −/− mice could be associated, at least partially, with the increased levels of GluR6 and GluR7 KA receptor subunits.

Whether PrPc participates in the modulation and expression levels of KA receptor subunits is unknown. However, many intracellular interactions have been described for PrPc, including synaptic proteins (e.g., Synapsin I), adaptor proteins (Grb2), or cytoskeletal proteins (PSD95/SAP90) (Sakudo et al., 2006). Although the functions of most of these interactions are obscure, most participate in cell survival and neurotransmission. In addition, PrPc-induced intracellular transmission acting via the Fyn and ERK1-2 intracellular cascade also controls a large number of intracellular effectors and neural functions (Chiarini et al., 2002; Monnet et al., 2004; Gavin et al., 2005, which might, in turn, regulate KA receptor expression or activity.

Moreover, our data also point to NMDA receptors participating in the increased neurotoxicity in KA-treated *Prnp*-deficient neurons. In fact, the participation of NMDA and non-NMDA receptors in KA-induced excitotoxicity has been described previously (Frandsen et al., 1989) and facilitated glutamatergic transmission associated with increased expression of N2RA and N2RB NMDA receptor subunits has been reported in *Prnp* −/− mice (Maglio et al., 2004). Decreased GABAa-mediated inhibition has been also reported in PrPc-mutant mice (Collinge et al., 1994) and it is well known that KA decreases GABAergic inhibition in neurons (Lerma 2006). In conclusion, our results, together with those reported above, indicate that factors involved in the onset of epileptogenic processes are dysregulated in *Prnp* −/− mice and suggest that increased levels of GluR 6-7 may participate in the susceptibility of *Prnp* −/− mice to kainic acid.

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